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# **THE ROLE OF SARCOPLASMIC CALCIUM IN SKELETAL MUSCLE TRAINING ADAPTATION**

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# THE ROLE OF SARCOPLASMIC CALCIUM IN SKELETAL MUSCLE TRAINING ADAPTATION THESIS FOR DOCTORAL DEGREE (Ph.D.)

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## ABSTRACT

Current research shows a clear correlation between strong mitochondrial capacity, healthy muscle and general public health. A sedentary lifestyle increases the risk of a whole host of so called 'western diseases', while an active lifestyle reduce the risk of said diseases. Thus, well-functioning muscles are a necessity for general health. So far endurance exercise is the most effective method to improve muscle function. This thesis will focus on the cellular mechanisms that regulate muscle performance and how these can be improved.

In the first study, we show that supplemented dietary nitrate enhances  $\text{Ca}^{2+}$  handling and submaximal force in mouse fast twitch muscle. Continuing this, in study two, we show that the increased submaximal force enhances voluntary activity in mice, presumably due to a shifted perceived effort of running. In study three we show that mild stress from cold exposure can enhance mitochondrial biogenesis resulting in improved fatigue resistance without exercise. The cold environment seems to induce a sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$  leak in the skeletal muscle. In study four we investigated why short (180s) high intensity interval training works better for enhancing endurance than regular low-intensity exercise. We show that oxidants formed during exercise causes ryanodine receptor modifications, which result in a SR  $\text{Ca}^{2+}$  leak and this in turn likely triggers transcription to improved mitochondrial capacity. In study five we show that inducing a mild SR  $\text{Ca}^{2+}$  leak, either with exercise or pharmacological tools, drive mitochondrial biogenesis. In study six we show that in a model of ageing, a degenerative mitochondrial problem causes myopathy via reduced SR  $\text{Ca}^{2+}$  release.

$\text{Ca}^{2+}$  is a central player in muscle function. This thesis shows that diet, exercise and age have the ability to affect skeletal muscle  $\text{Ca}^{2+}$  handling. Most importantly,  $\text{Ca}^{2+}$  signals can improve mitochondrial function, resulting in improved muscle function. However degenerative mitochondria causes reduced  $\text{Ca}^{2+}$  handling that leads to muscle weakness. This is one of the reasons an active lifestyle is so important for the elderly, because it improves the mitochondrial function rather than being degraded. Perhaps in the future, inducing a small SR  $\text{Ca}^{2+}$  leak could minimize some of the risks associated with sedentary lifestyle.

## LIST OF SCIENTIFIC PAPERS

- I. Hernández A, Schiffer TA, **Ivarsson N**, Cheng AJ, Bruton JD, Lundberg JO, Weitzberg E and Westerblad H (2012). Dietary nitrate increases tetanic  $[Ca^{2+}]_i$  in mouse fast-twitch muscle *J Physiol* **590**, 3575-3583.
- II. **Ivarsson N**, Schiffer TA, Hernández A, Lanner JT, Lundberg JO, Weitzberg E and Westerblad H. Dietary nitrate markedly improves voluntary running in mice. *In Manuscript*.
- III. Bruton JD, Aydin J, Yamada T, Shabalina IG, **Ivarsson N**, Zhang SJ, Wada M, Tavi P, Nedergaard J, Katz A and Westerblad H (2010). Increased fatigue resistance linked to  $Ca^{2+}$ -stimulated mitochondrial biogenesis in muscle fibers of cold-acclimated mice. *J Physiol* **588**, 4275-4288.
- IV. Place N, **Ivarsson N**, Venckunas T, Neyroud D, Brazaitis M, Cheng AJ, Ochala J, Kamandulis S, Girard S, Volungevičius G, Paužas H, Mekideche A, Kayser B, Martinez-Redondo V, Ruas JL, Bruton JD, Truffert A, Lanner JT, Skurvydas A and Westerblad H (2015) Ryanodine receptor fragmentation and sarcoplasmic reticulum  $Ca^{2+}$  leak after one session of high-intensity interval exercise. *Accepted Proc Natl Acad Sci USA*
- V. **Ivarsson N**, Cheng AJ, Bruton JD, Westerblad H and Lanner JT. (2015). Sarcoplasmic reticulum  $Ca^{2+}$  leak and increased endurance in mouse muscle. *In Manuscript*
- VI. **Ivarsson N**, Yamada T, Hernández A, Fhalström, A, Cheng, AJ, Zhang SJ, Bruton JD, Ulfhake B, Westerblad H (2012). Impaired mitochondrial respiration and decreased fatigue resistance followed by severe muscle weakness in skeletal muscle of mitochondrial DNA mutator mice *J Physiol* **590**, 6187-6197.

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## LIST OF ABBREVIATIONS

[Ca <sup>2+</sup> ] <sub>i</sub>	Cytosolic free [Ca <sup>2+</sup> ]
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	5'-adenosinemonophosphate-activated protein kinase- $\alpha$
ATP	Adenosine triphosphate
CaM	Calmodulin
CaMK	Calmodulin kinase
CaN	Calcineurin
COX1	Cytochrome c oxidase, subunit 1
CS	Citrate synthase
CSQ	Calsequestrin
DHPR	Dihydropyridine receptor
DMEM	Dulbecco's Modified Eagle's medium
EDL	Extensor digitorum longus
EMG	Electromyography
FAD	Flavin adenine dinucleotide
FDB	Flexor digitorum brevis
HAD	3-hydroxyacyl-CoA dehydrogenase
HIIT	High-intensity interval training
MDA	Malondialdehyde adducts
MVC	Maximal voluntary contraction
NAD <sup>+</sup> /NADH	Nicotinamide adenine dinucleotide
NFAT	Nuclear factor of activated T
P <sub>i</sub>	Inorganic phosphate
PGC-1 $\alpha$	Peroxisome proliferator-activated receptor $\gamma$ co-activator-1 $\alpha$
PLFFD	Prolonged low-frequency force depression
PolgA	mtDNA Polymerase $\gamma$ , subunit A
ROS/RNS	Reactive oxygen/nitrogen species
RyR	Ryanodine receptor
SERCA	Sarcoplasmic reticulum Ca <sup>2+</sup> ATPase
SOD	Superoxide dismutase
SR	Sarcoplasmic reticulum
TA	Tibialis anterior
TFAM	Mitochondrial transcription factor A
UCP	Uncoupling protein



# 1 INTRODUCTION

It is not only our intellect that separates human beings from other animal species. Although our sprint performance is rather pathetic compared to other species, even other primates; our ability for endurance running is unrivaled, even compared to most quadrupedal species (Bramble & Lieberman, 2004). Thus, we humans appear to have evolved to be on the move.

For the average human, skeletal muscle tissue represents approximately 40% of our body weight, and at rest is responsible for 20-30% of the metabolic rate (Zurlo *et al.*, 1990; Rolfe & Brown, 1997). In other words, our muscles are a big, and important, part of us. It might not be so surprising then that a sedentary lifestyle is associated with a host of so called “western world” diseases (e.g. obesity, metabolic syndrome, diabetes and cardiovascular disease) (Vincent *et al.*, 2007; Booth *et al.*, 2011; Lee *et al.*, 2014b). On the other hand, an active lifestyle (i.e. utilized muscle) promotes both mental and physical health (Vincent *et al.*, 2007). Utilizing our muscles, or ‘exercise’ is suggested as a must for the general population in order to reduce the risk of cardiovascular mortality (Lee *et al.*, 2014b). Even in treatment of diseases not directly related to our muscles (i.e. cancer), exercise improves the general quality of life outcome (Meneses-Echavez *et al.*, 2015).

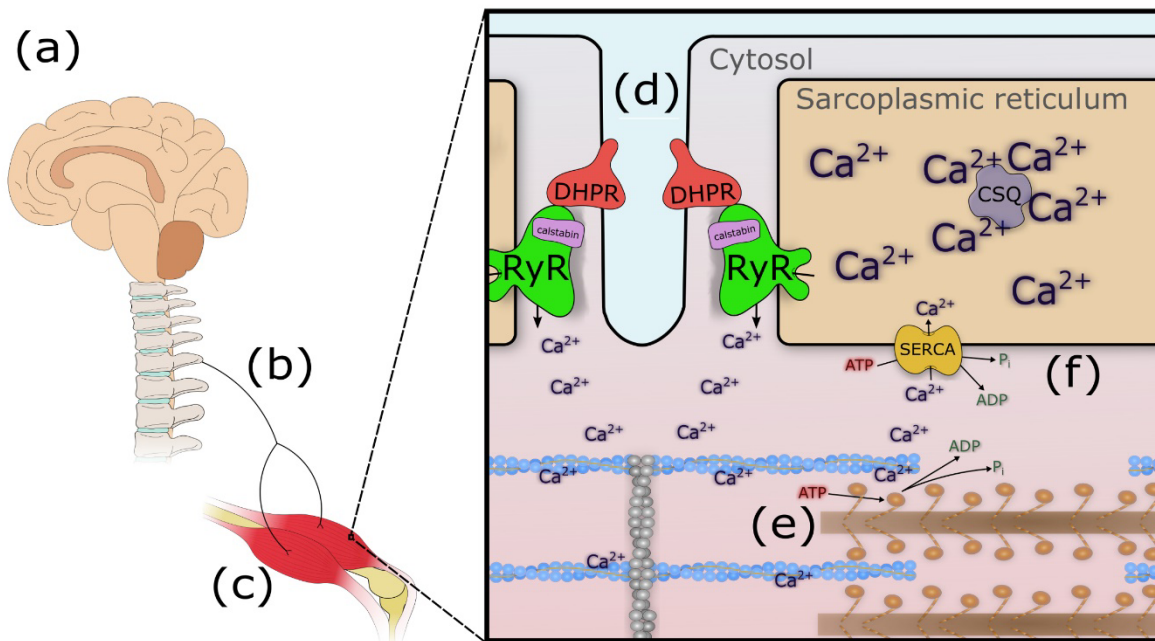
This thesis will focus on the cellular mechanisms that regulate muscle performance and how these can be improved.

## 1.1 MUSCLE ACTIVATION

Voluntary muscle activation is controlled by the prefrontal- and motor cortex via  $\alpha$ -motor neurons. Force production consequently depends both on the neural activation and the peripheral response to this activation (Fig 1). The relationship between  $\alpha$ -motor neurons firing frequency and muscle activation is not linear; the force-frequency relationship is steep at low stimulation frequencies (~10-30 Hz in human muscle) and reaches saturation at higher frequencies (>50 Hz) (Merton, 1954; Edwards *et al.*, 1977) (Fig 2). In mice, the relationship follows the same pattern, but plateaus >70Hz. Physical activity generally require low to moderate force and the firing frequencies of  $\alpha$ -motor neurons are therefore mostly set to be on the steep part of force-frequency curve (Marsden *et al.*, 1971; Grimby & Hannerz, 1977). Upon neural activation, an action potential propagates along the sarcolemma and t-tubular system where it activates the t-tubular voltage sensors, the dihydropyridine receptors (DHPR). DHPR mechanically activates the ryanodine receptors 1 (RyR1), located in the sarcoplasmic reticulum (SR) membrane (Rebbeck *et al.*, 2014).

The SR is a subcellular organelle which buffers  $\text{Ca}^{2+}$ . The great  $\text{Ca}^{2+}$  buffering capacity of SR is due to  $\text{Ca}^{2+}$  sequestering proteins, such as calsequestrin (CSQ) (Niki *et al.*, 1996; Murphy *et al.*, 2009). This buffering capacity results in a  $>10,000$  fold  $\text{Ca}^{2+}$  concentration gradient between SR and the cytosol (Toyoshima, 2008).

Voltage changes due to the action potential cause a structural change in DHPR which translates over to RyR1, opening the channel and  $\text{Ca}^{2+}$  is released to the intracellular space (Rebbeck *et al.*, 2014). This will result in a swift increase in the free cytosolic  $[\text{Ca}^{2+}]_i$  (Martonosi, 1984).



*Figure 1. Illustration of muscle activation, from prefrontal cortex via motor cortex (a) and the spinal cord (b) through  $\alpha$ -neurons to the neuromuscular endplate synapse (c). The depolarization generated moves across the sarcolemma down in the t-tubular network (d). The depolarization activates DHPR, which mechanically activates RYR1 in the SR membrane. SR  $\text{Ca}^{2+}$  release follows.  $[\text{Ca}^{2+}]_i$  then facilitates myosin-actin interaction which generates muscle force (e).  $\text{Ca}^{2+}$  is continuously pumped back to the SR via SERCA (f).*

### 1.1.1 Ryanodine receptor

There are three known isoforms of the ryanodine receptor, 1, 2 and 3. RyR1 is part of the skeletal muscle excitation-contraction coupling, RyR2 is found in heart, with similar function. RyR1 is a large ( $\sim 2$  MDa) complex built up of four identical 565kDa subunits (Lanner *et al.*, 2010; Zalk *et al.*, 2015). The complex is stabilized by the small protein calstabin1/FKBP12 (Ahern *et al.*, 1994; Zalk *et al.*, 2015). RyR is a passive ion channel,

which means that the rate of flow is determined by the  $\text{Ca}^{2+}$  gradient. However, the opening of the channel can be regulated by a host of interacting molecules and proteins. For RyR2, increased  $[\text{Ca}^{2+}]_i$  opens the channel (Bers, 2002). In skeletal muscle  $[\text{Ca}^{2+}]_i$  effect on RyR1 is less pronounced, however high concentrations can inhibit opening (Lanner *et al.*, 2010).  $\text{Mg}^{2+}$  is believed to compete for  $\text{Ca}^{2+}$  binding sites on RyR, the suggested consequence is an increased inhibition for RyR1 when both  $[\text{Ca}^{2+}]_i$  and cytosolic free  $[\text{Mg}^{2+}]$  is elevated (Meissner *et al.*, 1986; Lanner *et al.*, 2010). ATP, and other various forms of adenine nucleotides (ADP, AMP etc.), can potentiate RyR opening, with ATP being most effective (Meissner *et al.*, 1986; Lanner *et al.*, 2010). Calmodulin (CaM) is a  $\text{Ca}^{2+}$  binding protein also associated with RyR. In cardiac, CaM can shift the  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release; in skeletal muscle the role of CaM on RyR1 is less understood. Several phosphorylation sites can also be found on RyR. In cardiac,  $\beta$ -adrenergic stimulation can trigger protein kinase A (PKA) phosphorylation of RyR2, which increases the open probability of the channel (Lanner *et al.*, 2010). cGMP-dependent kinase (PKG) and  $\text{Ca}^{2+}$  induced calmodulin kinase (CaMK) can also phosphorylate certain sites on RyR2. These phosphorylation sites and kinases exist in skeletal muscle as well, however the little is known of the consequence of phosphorylated RyR1 (Lanner *et al.*, 2010).

## 1.2 FORCE GENERATION

The sarcomere is the functional force generating unit in skeletal muscle. In this structure  $[\text{Ca}^{2+}]_i$  will bind to troponin C, which sits in a complex with troponin T, I and tropomyosin on the actin filament. This will cause a movement of tropomyosin, which normally blocks the myosin binding site on actin. With actin exposed, the myosin head can form a cross-bridge structure with actin. ATP will bind to myosin. When ATP is converted to ADP and free inorganic phosphate ( $\text{P}_i$ ) energy is released which reangle the myosin head. Once the movement has occurred, myosin will release and form a new cross-bridge binding further up the actin filament. As long as  $\text{Ca}^{2+}$  is bound to troponin C, myosin will continue to cycle 'inward', shortening the sarcomere, and consequently the entire muscle cell (Gordon *et al.*, 2000).

$\text{Ca}^{2+}$  is continually pumped back to the SR by the sarcoplasmic reticulum  $\text{Ca}^{2+}$  ATPase (SERCA) in a process which requires the conversion of ATP to ADP and  $\text{P}_i$  (Inesi *et al.*, 2002; Toyoshima, 2008). The frequency by which RyR1 is opened dictates the levels of  $[\text{Ca}^{2+}]_i$ . Thus,  $[\text{Ca}^{2+}]_i$  influx is linear to stimulation frequency, however, cross-bridge formation will eventually reach saturation, explaining the frequency-force relationship (Fig 2).

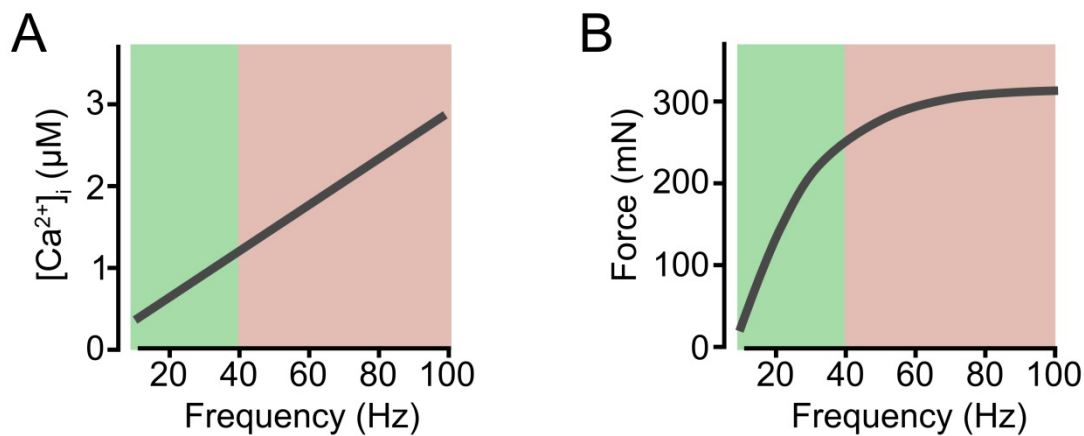


Figure 2. Graf depicting typical stimulation frequency - $Ca^{2+}$  release (A) and -force relationship (B) in mouse. Linear part of low frequency stimulation (normal everyday activity) highlighted green. Cross-bridge saturation highlighted with red.

### 1.3 FATIGUE AND ENDURANCE

Fatigue is a loose term usually attributed to a state of prolonged muscle weakness which limits a person's ability to perform physically demanding tasks. Not to be conflated with reduced maximal strength, which often is seen in various diseases and usually related to reduce expression, or post-translational modification of the myofibrillar proteins (Yamada *et al.*, 2014; Friedrich *et al.*, 2015).

Fatigue can be related to either peripheral problems (i.e. muscle activation) or central problems (e.g. reduced neural activity and mental effort). Peripheral fatigue can induce a prolonged low frequency force depression (PLFFD), which means that low intensity (~10-30 Hz) tasks require greater mental effort (Westerblad *et al.*, 1993). Physical activities generally require low to moderate forces and the firing frequencies of  $\alpha$ -motor neurons are therefore mostly set to be on the steep part of force-frequency curve (Marsden *et al.*, 1971; Grimby & Hannerz, 1977). Thus, small changes in mental effort affect SR  $Ca^{2+}$  release, which will have a large impact on submaximal force. This will have a large impact especially when the neuromuscular system is put under stress, e.g. during fatiguing exercise (Gandevia, 2001; Enoka *et al.*, 2011).

What determines the muscles capacity to perform repeated contractions (i.e. endurance)? Unsurprisingly, people with high oxidative capacity tend to have higher endurance (Holloszy & Coyle, 1984; Booth & Thomason, 1991). When comparing different muscle fiber types, type I muscle fibers can perform more repeated contractions compared to the type II fibers (Allen *et al.*, 2008b). Type I muscle fibers, also known as slow-twitch-, oxidative- or red muscle- fibers, naturally have greater amounts of mitochondria than type II fibers, also

known as fast-twitch-, glycolytic- or white- fibers. When we exercise, we increase the mitochondrial capacity in our muscle fibers (Holloszy & Coyle, 1984; Booth & Thomason, 1991). Therefore mitochondrial oxidative capacity in the muscle cell delimits the endurance, i.e. how intense and how many repeated contractions the muscle cell can sustain.

As I described earlier, the force generated by a muscle is related to amount of  $\text{Ca}^{2+}$  released from the SR. As RyR1 is a passive ion channel, the rate of  $\text{Ca}^{2+}$  flow is dependent on the  $>10,000$  fold  $\text{Ca}^{2+}$  concentration gradient between SR and cytosol (Toyoshima, 2008). SERCA, which actively pumps  $\text{Ca}^{2+}$  back to the SR is estimated to have the capacity to pump two  $\text{Ca}^{2+}$  ions per ATP (2 mol/mol) (Inesi *et al.*, 2002). The skeletal muscle is responsible for 20-30% of our metabolic rate at rest (Smith *et al.*, 2013). Therefore, a large proportion of the muscle energy is consumed by SERCA just in order to maintain the  $\text{Ca}^{2+}$  gradient, or baseline  $[\text{Ca}^{2+}]_i$  in between contraction as low as possible (Norris *et al.*, 2010; Smith *et al.*, 2013). This process generates heat (de Meis, 2001), and is a suggested mechanism of how skeletal muscle can participate in thermogenesis (Head *et al.*, 2015). Even in repeated submaximal isometric contractions (35% of maximal force), cycling of myosin only account for ~20% of total ATP consumption (Zhang *et al.*, 2006a). Thus, maintaining the  $\text{Ca}^{2+}$  gradient is very energy consuming.

ATP creates energy by cleaving of one  $\text{P}_i$  forming ADP. The sum of all this is; unless the metabolism of the muscle cell can keep up,  $\text{P}_i$  will accumulate. Muscle fatigue generated in single muscle fiber experiments with repeated contractions show that in the first set of contractions there is a decrease in  $\text{Ca}^{2+}$  sensitivity (i.e. force declines despite no changes in tetanic  $[\text{Ca}^{2+}]_i$ ). This first stage of fatigue is due to the accumulating  $\text{P}_i$ , preventing some cross-bridge formation. After the first couple of contractions the muscle enters a “sustainable”, or second stage, where neither  $\text{Ca}^{2+}$  nor force between contractions changes. The length of this stage correlates with the oxidative capacity of the muscle. Eventually force starts to decrease because SR  $\text{Ca}^{2+}$  release decreases (Allen & Westerblad, 2001; Allen *et al.*, 2008b). There are some theories as to why  $\text{Ca}^{2+}$  release decreases in the late stages of repeated contractions. For instance,  $\text{P}_i$  as a molecule can, when in contact with high concentrations of  $\text{Ca}^{2+}$ , form  $\text{CaP}_i$  crystals. It has therefore been suggested that high concentrations of  $\text{P}_i$ , which have accumulated during the repeated contractions, enter SR and form  $\text{CaP}_i$  and precipitate, reducing the available SR  $[\text{Ca}^{2+}]$  from being released (Allen *et al.*, 2008a, b). Formations of  $\text{CaP}_i$  in the SR would reduce the amount of releasable  $\text{Ca}^{2+}$ , and thus the cytosol/SR  $\text{Ca}^{2+}$  gradient.

Fatigue is not exclusively related to limitations of oxidative capacity. For instance, in classical experiments presented by Angelo Mosso in his book *La fatigue*, he showed that when a test subject was mentally fatigued (i.e. after giving a lecture in physiology), force diminished faster when performing repeated contractions (Mosso, 1904). Mental, or central, fatigue can thus generate the perception of muscle weakness.

Equally so, metabolic stress of the muscle can generate central fatigue. During physical exercise there is afferent sensory feedback, which adjusts the central motor drive to the metabolic state of the muscle, presumably to prevent peripheral fatigue (Gandevia, 2001; Amann, 2012).

The most effective way to counteract fatigue is increased muscle endurance, and so far, the most effective method of increased muscle endurance is endurance exercise (Holloszy & Coyle, 1984; Booth & Thomason, 1991). However, as I will discuss in this thesis, regular endurance exercise is not necessarily the only method to improve the muscles oxidative capacity.

## **1.4 METABOLISM**

ATP is supplied by either anaerobic process; or with oxygen utilization, aerobic processes. The anaerobic processes include phosphocreatine (PCr) buffer, which can resupply ATP by transferring its phosphate to ADP. PCr is a fast, but low capacity process, resulting in increasing levels of creatine and  $P_i$ . Glycolysis metabolizes glucose to lactic acid to reproduce ATP. Glycolysis can also be part of anaerobic metabolism. If oxygen is present, acetyl-CoA is formed instead of lactic acid. (Devlin, 2006).

Fatty acids are broken down to acetyl-CoA with  $\beta$ -oxidation. A four step process where at first flavin adenine dinucleotide (FAD) is used to dehydrate and create a double bond between carbon 2 and 3. The double bond is in then hydrated resulting in an OH attachment at C3. In the third step,  $\beta$ -hydroxyacyl CoA dehydrogenase (HAD) utilizes nicotinamide adenine dinucleotide ( $NAD^+$ ) to transform the OH on C3 into a double bounded oxygen. In the fourth and final step,  $\beta$ -ketothiolase cleaves the fatty acid between C2 and C3 forming one acetyl-CoA and a two carbon atom shorter fatty acid to be cycled through  $\beta$ -oxidation again (Devlin, 2006).

In the mitochondrial matrix, the enzyme citrate synthase (CS) utilize acetyl-CoA, generated through glycolysis or  $\beta$ -oxidation, together with oxaloacetate to form citric acid, initiating the

citric acid cycle. The citric acid cycle will generate NADH, which is used in the electron transport chain to produce ATP (Devlin, 2006).

Succinate and NADH generated in the citric acid cycle are used in the electron transport chain in order to pump  $H^+$  out from the mitochondrial matrix. Complex V acts as a hydroelectric dam, using the generated  $H^+$  gradient in a fashion which mimics the action of SERCA in reverse, as  $H^+$  passes through, the complex uses the energy to reattach  $P_i$  to ADP and form new ATP (Devlin, 2006).

## 1.5 MITOCHONDRIAL BIOGENESIS

Given the correlation between endurance and mitochondrial capacity; if we want to improve our endurance we need to find the method to increase the muscles capacity to resupply ATP.

The mitochondrion is unique as a subcellular organelle because it carries its own genetic code, mitochondrial DNA (mtDNA). The nucleus can trigger mtDNA replication by producing mitochondrial transcription factor A (TFAM, sometimes also abbreviated mtTFA) and mitochondrial transcription factor B2 (TFB2M). These transcription factors together with mtDNA polymerase are transported into the mitochondrial matrix, where mtDNA replication occurs (Gensler *et al.*, 2001; Falkenberg *et al.*, 2007).

The number of mitochondria is not as important for oxidative capacity compared to the enzyme content or volume of each mitochondrion. The mitochondrial DNA encodes 37 unique genes, however, only 13 are actual proteins, and those only form a small part of the >1000 peptides partaking in oxidative metabolism (Taylor & Turnbull, 2005). The gene regulation for enhances oxidative capacity is more complex than just two transcription factor and a polymerase.

Peroxisome proliferator-activated receptor  $\gamma$  co-activator-1 $\alpha$  (PGC-1 $\alpha$ ) has been identified to play a central role in gene expression of for the majority of factors necessary for mitochondrial biogenesis and increased oxidative capacity (Wu *et al.*, 1999; Baar *et al.*, 2002). Overexpression of PGC1 $\alpha$  increases the volume and number of mitochondria (Lehman *et al.*, 2000). Mice where PGC-1 $\alpha$  is selectively over-expressed in skeletal muscle become fatigue resistant (Lin *et al.*, 2002). Conversely, mice with PGC-1 $\alpha$  knocked out have less metabolic capacity (Lin *et al.*, 2004). Importantly, PGC1 $\alpha$  is up regulated in humans after performed endurance exercise (Norrbon *et al.*, 2004).

Therefore, PGC-1 $\alpha$  seems like a major target candidate in order to increase mitochondrial capacity. Intriguingly, in experiments where baseline  $[Ca^{2+}]_i$  has been pharmacologically

increased, PGC1 $\alpha$  also increases (Wu *et al.*, 2002). And this is several days later followed by increases in mitochondrial enzymes (Ojuka *et al.*, 2003). In a system such as the skeletal muscle where  $[Ca^{2+}]_i$  oscillate 20 fold (0,05 - 1  $\mu$ M) simply by us taking a walk makes this signaling pathway intriguing.

The  $[Ca^{2+}]_i$  induced mitochondrial biogenesis is proposed to occur via  $Ca^{2+}$ -calmodulin-dependent protein kinase II (CaMKII) and calcineurin (CaN) signaling (Wu *et al.*, 2002; Tavi & Westerblad, 2011). An excess  $[Ca^{2+}]_i$  activates CaMKII and/or CaN, which dephosphorylate nuclear factor of activated T (NFAT). Dephosphorylated NFAT moves into the nucleus and stimulates transcription of several transcription factors subsequently increasing PGC-1 $\alpha$  transcription (Tavi & Westerblad, 2011). For instance, consecutively activated CaN increases gene expression and muscle phenotype towards oxidative (type I) muscle fibers (Naya *et al.*, 2000; Wu *et al.*, 2001). Conversely, mice with CaN knocked out had decreased nuclear NFAT and less type I muscle fiber contents (Parsons *et al.*, 2003).

This indicates that  $Ca^{2+}$  mediated CaN activity is important for enhanced oxidative capacity. However, CaN have to be activated for a prolonged period (hours) in order for a sufficient gene transcription to take place (Tavi & Westerblad, 2011). The burst of increased  $[Ca^{2+}]_i$  during a contraction is too short to have a lasting effect. So the question remains, where does the  $Ca^{2+}$  necessary for CaN and CaMKII activation come from? The answer to this question will be addressed in this thesis.

## 1.6 OXIDATIVE STRESS

“Free radicals” or reactive oxygen/nitrogen species (ROS/RNS) are reactive molecules which react and modify other molecules in the cell, primarily proteins, but also lipids and DNA. Superoxide ( $O_2^{\bullet-}$ ) is produced during mitochondrial oxidative phosphorylation but also by NADPH-oxidases. Nitric oxide ( $NO^{\bullet}$ ) radicals are formed by the enzyme nitric oxide synthase. Higher demand on ATP production increases the risk of  $O_2^{\bullet-}$  forming. Therefore, increased ROS/RNS production occurs as a consequence of the increased metabolic stress during exercise. Under normal conditions several endogenous antioxidant systems act to neutralize increased ROS (superoxide dismutase (SOD), thioredoxin and glutathione reductase) (Powers & Jackson, 2008). Strong oxidative capacity is associated with good antioxidant capacity. Endurance exercise in itself can increase these different endogenous antioxidant systems (Ji, 2002).

Uncontrolled free radicals induce certain modifications on proteins.  $O_2^{\bullet-}$  can react with carboxyl groups on proteins forming what is called carbonylation. They can also interact with



lipids which cause the reactive molecule malondialdehyde to be formed. This in turn interacts with the amino acid lysine on proteins and form malondialdehyde adducts (MDA) (Stadtman & Levine, 2003). NO• can oxidize cysteine residues on proteins by forming reversible S-nitrosylation modification to the sulfur atom on cysteine. The sulfur part of cysteine is particularly important in protein folding, since it often forms so called sulfur bridges (S-S) to other cysteine residues (Hess *et al.*, 2005). NO• and O<sub>2</sub>• can react with each other and form peroxynitrate (ONOO<sup>-</sup>). ONOO<sup>-</sup> in turn can interact with tyrosine and form 3-nitrotyrosin modifications (Szabó *et al.*, 2007).

Most protein-protein interactions rely on just a few amino acids for attraction. All these modifications to some degree alter the general structure and thus function of proteins (Stadtman & Levine, 2003). RyR1 is for instance shown to be very susceptible to ROS modification. A consequence of a modified RyR1 is a 'leaky' channel, with subsequent prolonged increase in baseline [Ca<sup>2+</sup>]<sub>i</sub>. (Bellinger *et al.*, 2008; Bellinger *et al.*, 2009; Andersson *et al.*, 2011; Lanner *et al.*, 2012; Yamada *et al.*, 2014).

## 1.7 CYTOSOLIC CA<sup>2+</sup>, MITOCHONDRIA AND DISEASE

Muscle weakness can be the result of mitochondrial dysfunction (i.e. mitochondrial myopathy). Generally mitochondrial myopathies are caused by genetic factors (Luft *et al.*, 1962; Larsson & Oldfors, 2001). However, muscle weakness and fatigability are also common traits of ageing. The link between dysfunctional mitochondria and ageing can be seen in a mouse model where defective proof reading subunits in mtDNA polymerase  $\gamma$ , subunit A (PolgA) have been introduced. This result in degenerating mitochondria, as each new mtDNA contains more errors (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005).

In another mitochondrial myopathy model, where *TFAM* gene has been knocked-out from adult type II muscle fibers, excess [Ca<sup>2+</sup>]<sub>i</sub> enters the mitochondria, resulting in down regulation of SR Ca<sup>2+</sup> buffering protein CSQ and subsequent decrease of tetanic [Ca<sup>2+</sup>]<sub>i</sub> (Wredenberg *et al.*, 2002; Aydin *et al.*, 2009). If the mechanism by which mitochondria can absorb Ca<sup>2+</sup> is blocked, the reduction in SR Ca<sup>2+</sup> release and muscle myopathy is mitigated (Gineste *et al.*, 2015), indicating that excess [Ca<sup>2+</sup>]<sub>i</sub> can negatively affect the mitochondria.

Defective RyR1 or 'leaky' RyR1 channels, resulting in increased baseline [Ca<sup>2+</sup>]<sub>i</sub>, are implicated in several pathological states, including inflammatory disorders (Yamada *et al.*, 2014), heart failure (Reiken *et al.*, 2003), inherited conditions such as malignant hyperthermia (Lanner *et al.*, 2012) and Duchenne muscular dystrophy (Bellinger *et al.*,

2009). Normal ageing also seems to negatively affect the stability of RyR (Andersson *et al.*, 2011; Umanskaya *et al.*, 2014).

Muscle function is tied to effective SR  $\text{Ca}^{2+}$  release. Mitochondrial myopathies, such as the TFAM KO model, indicate that that not only is the health of the mitochondria important for normal muscle function, but excess  $[\text{Ca}^{2+}]_i$  affect the health of the mitochondria. This is rather interesting with the evidence that  $\text{Ca}^{2+}$  induces mitochondrial biogenesis. It appears that we have two rather conflicting sides on whether excess  $[\text{Ca}^{2+}]_i$  is good or bad. On one hand,  $\text{Ca}^{2+}$  can stress the mitochondria resulting in muscle weakness and heart failure; on the other hand,  $\text{Ca}^{2+}$  can promote mitochondrial biogenesis.

## 2 AIMS

The aims of this thesis are the following:

- Can muscle function be improved without exercise? We focused on the notable influence of dietary nitrate on athletic performance. Study I and II investigate how nitrate alters  $\text{Ca}^{2+}$  handling and how this in turn affects the perceived effort of physical activity.
- What is the initial signaling towards improved muscle endurance? In paper III, IV and V we investigate how skeletal muscle responds to various intense forms of metabolic stress and repeated contractions.
- What central role does the mitochondrion play in responding/regulating  $\text{Ca}^{2+}$  handling in the skeletal muscle? In paper VI we investigated how deteriorating mitochondria leads to myopathy.

## 3 METHODS

### 3.1 MOUSE STUDIES

#### 3.1.1 Ethical approval

Mice were used in all included studies. All animal experiments complied with the Swedish Animal Welfare Act, the Swedish Welfare Ordinance, and applicable regulations and recommendations from Swedish authorities. All studies included in this thesis were approved by the Stockholm North Ethical Committee on Animal Experiments.

Mouse strains used:

- 7-15 weeks old male C57Bl/6: Paper I, II, V
- 10 weeks old female C56Bl/6 Paper IV
- Genetically modified uncoupling protein 1 (UCP1) (-/-) or UCP1 (+/+): Paper III
- Mice strain containing deficient PolgA: Paper VI

#### 3.1.2 Housing

Animals were housed in a dedicated animal facility with a 12h/12h day/night cycle and regulated ~24°C room temperature. No more than 5 mice per cage. Food and water was supplied *ab libitum*. In paper III some mice were housed at a lower temperature of at first ~18°C and during experiment ~4°C.

#### 3.1.3 Nitrate feeding

Experiments in paper I and II; all mice were given food controlled for low nitrate content one week before treatment. Thereafter, one group of mice was given 1 mM NaNO<sub>3</sub> dissolved in distilled water. For comparison, this is equivalent to a human drinking two 70 ml shots of concentrated beetroot juice, a nitrate-source often used in human trials (Jones, 2014). Controls were provided distilled water without nitrate.

#### 3.1.4 Voluntary running

For paper II, IV and V; animals were individually housed in cages equipped with a wireless low profile running wheel (ENV-044 Med-Associates, St Albans City, VT) and running distance was continuously measured for the duration of the experiment. Sedentary control mice were housed individually in cages with locked running wheels.

### 3.1.5 Mouse endurance test

For paper II and V; mouse endurance was assessed using an animal treadmill (Exer 3/6, Columbus instruments, Columbus, OH). The mice were habituated to the treadmill one hour (50 min stationary and 10 min walking at  $10 \text{ m} \cdot \text{min}^{-1}$ ) a day for four days prior to the exhaustion test. During the test mice ran at  $25^\circ$  uphill. An initial 10 min warm-up ( $10 \text{ m} \cdot \text{min}^{-1}$ ) was followed by gradual increases of  $2 \text{ m} \cdot \text{min}^{-1}$  every 2 minutes. Exhaustion was determined as the time when the mouse had withstood three mild electric shocks (164 V, 0.1 mA, 2 Hz) without attempts to continue running. Method based on (Kemi *et al.*, 2002) showing mouse  $\text{VO}_2$  consumption increases until the mice reach 1:1  $\text{O}_2/\text{CO}_2$  exchange ratio, where the mice becomes exhausted and stop running.

### 3.1.6 Whole muscle force measurements

Mouse *extensor digitorum longus* (EDL), *soleus* and *flexor digitorum brevis* (FDB) muscles were removed and mounted at optimal length between a force transducer and an adjustable hook in a chamber filled with Tyrode solution (mM): NaCl, 121; KCl, 5.0;  $\text{CaCl}_2$ , 1.8;  $\text{MgCl}_2$ , 0.5;  $\text{NaH}_2\text{PO}_4$ , 0.4,  $\text{NaHCO}_3$ , 24.0; EDTA, 0.1; glucose, 5.5 and fetal calf serum (0.2%). The solution was bubbled with 95%  $\text{O}_2$ , 5%  $\text{CO}_2$  and kept at a temperature of  $30^\circ \text{C}$ . The force-frequency relationship was established by producing 300 ms contractions at 1-150 Hz stimulation frequencies at 1 minute interval.

The the single digit FDB muscles in paper IV were stimulated with a simulated 'Wingate' protocol of 100 Hz trains (250 ms duration, 2 per second for 30 seconds) followed by 4 minutes recovery. The stimulation protocol was repeated for a total of 6 times. Muscles were allowed to recover for 5 min, 30 min or 3 hours.

### 3.1.7 Force and $[\text{Ca}^{2+}]_i$ measurements in single muscle fibers

Intact, single muscle fibers were dissected from mouse FDB muscles as described elsewhere (Lännergren & Westerblad, 1987). The isolated fiber was mounted in a stimulation chamber at optimum length and superfused with Tyrode solution. The solution was bubbled with 5%  $\text{CO}_2$ –95%  $\text{O}_2$ , which gives an extracellular pH of 7.4. Experiments were performed at room temperature ( $\sim 25^\circ \text{C}$ ). Tetanic stimulation was achieved by supramaximum current pulses (duration 0.5 ms) delivered via platinum plate electrodes lying parallel to the muscle fiber.

The fluorescent  $\text{Ca}^{2+}$  indicator indo-1 (Invitrogen/Molecular probes, Carlsbad, CA, USA) was microinjected into the isolated fiber. The fiber was allowed to rest for at least 30 min after being injected with indo-1. It was then stimulated by individual 350 ms stimulation

trains at 10 to 150 Hz given at 1 min intervals. Tetanic force was measured as the mean over 100 ms where force was maximal.

The fluorescence of indo-1 was converted to  $[Ca^{2+}]_i$  using an intracellular established calibration curve (Andrade *et al.*, 1998). Tetanic  $[Ca^{2+}]_i$  was measured as the mean indo-1 fluorescence during tetanic stimulation trains, and basal  $[Ca^{2+}]_i$  as the mean over ~200 ms before stimulation trains.

In order to measure fiber endurance (paper III and V); fatigue was induced by repeated tetanic stimulations (70 Hz, 300 ms duration) given at 2 s intervals until force reached 40% of the initial.

### 3.1.8 Single fiber dissociation

Mouse FDB muscles were incubated at 37°C for 1 hour in 0.3% collagenase type I (Sigma, St Louis, MO, USA) in Dulbecco's Modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Muscles were thereafter transferred to 3 ml of DMEM and triturated to dissociate individual muscle fibers. Approximately 200  $\mu$ l of the muscle fiber suspension was loaded on laminin (Sigma) coated glass-bottom Petri dishes (MatTek, Ashland, MA, USA) and allowed to attach for five minutes. Additional 3 ml DMEM was added and the dishes were placed in an incubator at 37°C.

### 3.1.9 Measurements of mitochondrial ROS production.

Dissociated single FDB fibers were loaded in 5  $\mu$ M of the fluorescent mitochondrial ROS indicator Mitosox Red (Invitrogen) in Tyrode for 15 min at room temperature, followed by washout.

In Paper IV the fibers were then stimulated with the stimulated 'Wingate' protocol. Images were obtained before, 5 minutes and 10 min after stimulation.

In Paper VI images were taken before and after incubation with 1mM  $H_2O_2$  in order to inhibit SOD2 activity.

Confocal images were obtained by excitation at 488 nm and measuring the emitted light at 585 nm. Changes in fluorescence intensity were calculated in each fiber by dividing post-intervention signals with the pre-intervention signal ( $F/F_0$ ).

### 3.1.10 Isolation of mitochondria

In Paper II, *gastrocnemius* muscle tissue was weighed and homogenised in ice-cold isolation medium containing (mM): sucrose, 100; KCl, 100; Trizma HCl, 50; KH<sub>2</sub>PO<sub>4</sub>, 1; EGTA, 0.1; and BSA, 2% (w/v). The homogenized sample volume was adjusted to 3 ml isolation medium and centrifuged at 700 g for 10 min (4°C). The supernatant containing the mitochondria was transferred to 1.5-ml Eppendorf tubes and centrifuged at 10,000 g for 10 min. The pellet was then carefully washed in isolation medium, and a buffy coat of extra mitochondrial debris was removed. The mitochondrial pellet was resuspended and centrifuged at 7,000 g for 5 min. The pellet was again washed and diluted in 0.6 µl preservation medium (mM): EGTA, 0.5; MgCl<sub>2</sub>, 3; K-lactobionate, 60; taurine, 20; KH<sub>2</sub>PO<sub>4</sub>, 10; HEPES, 20; sucrose, 110; histidine, 20; vitamin E succinate, 20 and (µM): glutathione, 3; leupeptin, 1; glutamate, 2; malate, 2; BSA 1 g/L and Mg-ATP 2mM/mg tissue sample.

### 3.1.11 Respirometry

Mitochondrial respiration was analysed, in paper II, by high-resolution respirometry in a 2-channel titration injection respirometer at 37°C (Oxygraph; Oroboros, Innsbruck, Austria), using respiration medium (mM): EGTA, 0.5; MgCl<sub>2</sub>, 3; K-lactobionate, 60; taurine, 20; KH<sub>2</sub>PO<sub>4</sub>, 10; HEPES, 20; sucrose, 110; and BSA 1 g/L. Respiratory control ratio (RCR) is calculated as the ratio between respiration with saturating amounts of substrates and ADP (state 3) and respiration with substrates when all ADP has been phosphorylated to ATP (state 4). Mitochondrial efficiency was assessed in the presence of ATP (2 mM) by infusion of non-saturating level of ADP with a microdialysis pump through high-pressure hosing. This method has been described in detail elsewhere (Gnaiger *et al.*, 2000). The ADP infusion rate was set to ~50% of maximal state 3 respiration and was initiated at an oxygen pressure of ~10 kPa. Mitochondrial efficiency (P/O) was assessed from the rate of infused ADP divided by the oxygen consumed during steady state at the last minutes of infusion. Correction was made for the amount of oxygen added to the respiration medium by the infused ADP solution.

## 3.2 HUMAN EXPERIMENTS

### 3.2.1 Human ethics

The human studies in paper IV were approved by the local Ethics Committees of Lithuania and Switzerland and performed in accordance with the Helsinki declaration. Each subject gave written informed consent before participation.

Data were obtained from four groups of male subjects.

- Recreationally active performing three sets of high intensity cycling bouts
- Recreationally active performing six sets of high intensity cycling bouts
- Recreationally active performing a marathon
- Elite athletes performing six sets of high intensity cycling bouts

Participants refrained from physical activity and caffeine consumption for 24h and 12h before the experimental session, respectively.

All participants were familiarized to the training session a few days before each experimental session. At the end of the familiarization session, maximal oxygen uptake ( $\text{VO}_{2\text{max}}$ ) was measured on a cycle ergometer using a standard incremental protocol.

### 3.2.2 High intensity interval training

Our high intensity exercise model comprised repeated Wingate tests of 30 s all-out cycling bouts at 0.7 Nm/kg body weight on a cycle ergometer, with 4 min rest between tests. Each experiment was preceded by a standard warm-up on the cycle ergometer (~5 min at 100 W). In initial experiments recreationally active subjects ( $n = 10$ ) performed  $3 \times 30$  s all-out efforts. In subsequent experiments, recreationally active subjects ( $n = 18$ ) and elite endurance athletes ( $n = 14$ ) performed  $6 \times 30$  s all-out efforts. The group of elite endurance athletes consisted of 7 mid- or long-distance runners and 7 road cyclists and all of them were at least competitive at the national level.

### 3.2.3 Marathon running

Eight recreationally active subjects participated in a marathon-distance outdoor running race. These subjects performed regular endurance training at a recreational level. During the race they were encouraged to perform their absolute best. The mean time to complete the marathon race was  $206 \pm 11$  min (mean  $\pm$  SEM).

### 3.2.4 Muscle force

Neuromuscular function of the knee extensors of the dominant leg was tested before and up to 24 hours after exercise. The tests consisted of a 5 s maximal voluntary contraction (MVC) followed by supramaximal electrical femoral nerve stimulation at 2 s intervals: paired stimuli at 100 Hz, 10 Hz, and single stimulus to obtain the M wave. Alternatively, evoked force from the knee extensors was measured during supramaximal tetanic stimulation delivered through surface electrodes with one electrode placed just above the patella and the other covering a large portion of the quadriceps muscle belly in the proximal third of the thigh (Kamandulis *et al.*, 2010). Isometric MVC force was considered as the peak force attained during the

voluntary contraction performed at a given time point. Pre- and post-exercise amplitude of the 10 Hz and 100 Hz paired stimuli as well as the first derivative of the force signal in response to a single stimulus (i.e. rate of force development of the twitch) were quantified to assess contractile alterations after exercise. Paired stimuli at 100 Hz were given superimposed on and immediately after MVCs to assess the voluntary activation level (VAL), which was used as an index of central fatigue and assessed as:

$$\text{VAL} = (1 - (\text{superimposed doublet force} / \text{potentiated doublet force})) \times 100$$

### 3.2.5 Analyses of EMG signals.

Electromyographic (EMG) activity of the dominant knee extensors *vastus lateralis* was recorded from the recreationally active group performing 6 repeated high intensity interval cycling tests (see Paper IV, SI appendix for more detail).

### 3.2.6 Muscle biopsies.

Needle biopsies were taken from the non-dominant *vastus lateralis* muscle before, and ~10 min (1 hour after marathon running) and 24 hours after exercise, using previously described and validated procedures (Magistris *et al.*, 1998). Briefly, after skin sterilization and local anesthesia, a 1–2-mm-long skin cut was made with the tip of a scalpel. Biopsies were collected using an automatic biopsy device (Bard Biopsy Instrument, Bard Radiology, Covington, GA). A 14-gauge disposable needle was then inserted through the cut, perpendicular to the muscle fibers, until the fascia was pierced. Two or three samples (~15 mg each) were collected from one puncture site at each time point. Muscle samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis.

### 3.2.7 Skinned fibers

Human muscle biopsy samples from four of the recreationally active subjects who performed the six high intensity interval exercises (in Paper IV) were extracted and placed in relaxing solution at 4°C. Bundles of ~50 fibers were dissected free and tied to glass capillary tubes at slightly stretched lengths. The muscle bundles were then treated with skinning solution (relaxing solution containing glycerol; 50:50 v/v) for 24 hours at 4°C, after which they were transferred to -20°C. Subsequently they were treated with sucrose and snap frozen in liquid nitrogen-chilled propane and stored at -160°C.

Skinned fiber force measurements were performed as described elsewhere (Larsson & Moss, 1993). In brief, on the day of an experiment, a fiber bundle was de-sucrosed and fiber segments extracted; a total of 15-17 muscle fibers per subject were analyzed. A fiber segment



1 to 2 mm long was mounted between a force transducer and a lever arm system. The system was placed on the stage of an inverted microscope and the sarcomere length was set to 2.75-2.85  $\mu\text{m}$  (Larsson & Moss, 1993). The maximal force generation was measured at 15°C as the difference between the steady-state isometric force in the activating solution and the resting force in the relaxing solution. Relaxing and activating solutions contained (in mM): Mg-ATP, 4; free  $\text{Mg}^{2+}$ , 1; imidazole, 20; EGTA, 7; creatine phosphate, 14.5 and KCl to adjust the ionic strength to 180 mM (pH 7.0). The free  $[\text{Ca}^{2+}]$  was set to 32  $\mu\text{M}$  and 1 nM for the activating and relaxing solution, respectively (Fabiato, 1988). The myofibrillar  $\text{Ca}^{2+}$  sensitivity was assessed by mixing the activating and relaxing solution and hence measure the force at different free  $[\text{Ca}^{2+}]$ . Curve-fitting was used to establish the  $[\text{Ca}^{2+}]$  required to obtain 50% of the maximal force ( $[\text{Ca}^{2+}]_{50}$ ).

After the mechanical measurements, each fiber segment was placed in urea buffer (120 g urea, 38 g thiourea, 70 ml  $\text{H}_2\text{O}$ , 25 g mixed bed resin, 2.89 g dithiothreitol, 1.51 g Trizma base, 7.5 g SDS, 0.004 % bromophenol blue) in a plastic micro-centrifuge tube and stored at -160°C. The myosin heavy chain (MyHC) isoform composition of fibers was determined by 6% SDS-PAGE. The acrylamide concentration was 4% (wt/vol) in the stacking gel and 6% in the running gel, and the gel matrix included 30% glycerol. Sample loads were kept small (equivalent to approximately 0.05 mm of fiber segment) to improve the resolution of the myosin heavy chain bands (types I, IIa and IIx). Electrophoresis was performed at 120 V for 24 h with a Tris-glycine electrode buffer (pH 8.3) at 15°C. The gels were silver-stained and subsequently scanned in a soft laser densitometer with a high spatial resolution (50  $\mu\text{m}$  pixel spacing) and 4096 optical density levels.

### **3.3 BIOCHEMISTRY**

#### **3.3.1 Protein extraction**

Muscle samples were homogenized with a ground glass homogenizer in ice-cold homogenisation buffer at pH 7.4 (20  $\mu\text{l}$  per mg wet weight) consisting of (mM): Hepes, 20; NaCl, 150; EDTA, 5; KF, 25;  $\text{Na}_3\text{VO}_4$ , 1; and 20% glycerol, 0.5% Triton X-100, and protease inhibitor cocktail (Roche, Basel, Switzerland) 1 tablet/50 ml. The homogenate was centrifuged at 700 g for 10 min at 4°C. Protein content of the supernatant was determined using the Bradford assay (#500-0006, Bio-Rad, Hercules, CA, USA).

#### **3.3.2 Western blot and immunoprecipitation**

For western blot, samples homogenates were diluted 1:1 in Laemmli buffer (Bio-Rad) with 5% 2-mercaptoethanol and heated to 95 °C for 5 min.

For immunoprecipitation (IP) (Paper IV and V), 1 µg anti-RyR1 (ab2868, Abcam, Cambridge, MA, USA) was bound to 12µl G-protein Dynal<sup>®</sup> magnetic beads (Invitrogen) per manufacturer's instructions. Samples were diluted to 0.5 µg/µl in ice-cold RIPA buffer pH 7.5 consisting of (mM): Tris HCl, 10; NaCl, 150; NaF, 5; Na<sub>3</sub>VO<sub>4</sub>, 1; and 1% Triton X-100, and protease inhibitor cocktail (Roche) 1 tablet/50 ml. The samples were then mixed with antibody-bead complex and incubated under rotation at 4°C overnight. The samples were gently washed 4 times with RIPA buffer. Peptides were then eluted from the beads with 50µl Laemmli buffer (Bio-Rad) with 5% 2-mercaptoethanol and heated to 95 °C for 5 min.

10 µg protein or 5 µl (mouse), 15 µl (human) IP eluate was run on a 4–12% precast Bis–Tris gel (NuPAGE, Invitrogen) and transferred (100V over 180 minutes) onto polyvinylidene fluoride membranes (Immobilon FL, Millipore, Billerica, MA, USA). Membranes were blocked with Li-Cor Blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) followed by incubation overnight at 4°C with the primary antibodies diluted in blocking buffer. See corresponding paper for complete list of antibodies

Membranes washed after incubation with primary antibody and incubated with secondary antibody IRDye 680-conjugated donkey anti-mouse IgG and IRDye 800-conjugated donkey anti-rabbit IgG (926-68072, 926-32213, LI-COR Biosciences) for 1 hour at room temperature. Immunoreactive bands were visualized using infrared fluorescence (IR-Odyssey scanner, LI-COR Biosciences). Band density was established using Image Studio v 2.0.38 (LI-COR Biosciences). Equal protein loading to each lane was also verified with Coomassie protein staining of membranes (Bio-Rad). For IP, the band density of RyR1 was used as loading control.

### 3.3.3 Enzymatic activity

Muscles samples were homogenized with a ground glass homogenizer in ice-cold buffer (50 µl (mg wet weight)<sup>-1</sup>) consisting of (mM): Tris, 50; sodium citrate, 5; MnCl<sub>2</sub>, 0.6; cysteine, 1; 0.05% (v/v) Triton X-100 (pH 7.4). The homogenate was centrifuged for 1 min at 1400 g (4°C) and aliquots of the supernatant were frozen for citrate synthase and β-hydroxyacyl-CoA dehydrogenase (HAD) analyses. Citrate synthase and HAD activities were analyzed with standard spectrophotometric techniques (Bass *et al.*, 1969). The activities were measured at room temperature under conditions that yielded linearity with respect to extract volume and time. The supernatant protein content was determined using the Bradford assay (BioRad, UK) and activities were adjusted for protein content.

### 3.3.4 Calpain activity.

The calpain activity assay kit (ab65308, Abcam) was used to determine calpain activity. In brief, single digit mouse FDB muscle were weighed and homogenized in 100  $\mu$ l of the supplied extraction buffer. Subsequently 85  $\mu$ l samples were loaded to a 96 well black coated polystyrene plate. For positive control, 2  $\mu$ l of supplied activated calpain was loaded to one well; for negative control, the supplied calpain inhibitor was added to one of the stimulated samples. After addition of reaction buffer and substrate, the plate was incubated for 60 min at 37 °C. After incubation the relative fluorescence units (RFU) of each well was measured with excitation at 380nm and emission at 485nm. Signals were then normalized to the wet weight of each FDB digit.

### 3.3.5 Immunohistochemistry

Dissociated fibers, as described above, or dissected FDB bundles were fixed with 0.4% paraformaldehyde for 30 min. Fixed single fibers or fiber bundles were permeabilized by 0.3% Triton X-100 in PBS solution. Dishes were rinsed with PBS and blocked with 3% bovine serum albumin. Fibers were then incubated with 1:150 rabbit anti-RyR antibody (#5029, gift from Prof Andrew Marks, Columbia University) and 1:150 mouse anti-L type  $\text{Ca}^{2+}$  channel DHPR alpha-2 subunit (ab2864, Abcam) at 4°C overnight. Dishes were washed with PBS and incubated with 1:100 donkey anti-rabbit Alexa Fluor 488 (A21206, Invitrogen) and 1:100 goat anti-mouse Alexa Fluor 568 (A11031, Invitrogen) for one hour at room temperature. Images of longitudinal thin sections of stained cells were obtained with laser confocal microscopy. Excitation was at 488 and 568 nm, and the emitted light was collected through 522- and 605-nm narrowband filters.

### 3.3.6 Glucose uptake

After prolonged incubation, muscles were transferred to vials containing 1.5 ml Tyrode solution with 2mM pyruvate replacing glucose. 1 mM radiolabelled 2-deoxyglucose and inulin were added followed by a 20 minute incubation in a shaking water-bath (110 oscillations min<sup>-1</sup>, 35°C, air phase in vial was continuously gassed with 95% O<sub>2</sub>–5% CO<sub>2</sub>) after which the muscles were frozen in liquid nitrogen. Frozen muscles were later dissolved in 1N NaOH. Dissolved solution was then added to scintillation liquid and  $\beta$ -radiation was assessed with a scintillation counter.

### 3.3.7 mRNA analyses.

Total RNA was obtained from skeletal muscle biopsies combining Isol-RNA Lysis Reagent (5 PRIME) and mechanical homogenization in Tissue Lyser II (Quiagen). After treating

mRNA with DNase (Invitrogen), 500 ng of cDNA were prepared according to the High-Capacity cDNA Reverse Transcription kit protocol (Applied Biosystems) and then diluted to a final volume of 180  $\mu$ l. Real-time determination of transcript abundance was SYBR-Green-based using 1  $\mu$ l of cDNA per reaction (approx. 2,78ng of cDNA) on a 5 :1 final volume.

Values were calculated as relative to the transcript abundance of hypoxanthine guanine phosphoribosyl transferase (HPRT), which showed no difference between groups and was therefore used as housekeeping gene (for complete list of primers see Paper IV, SI appendix).

### 3.3.8 Statistics

Data are presented as mean  $\pm$  SEM except when otherwise stated. Statistical significant changes were assessed using either unpaired, paired Students t-test or analysis of variance (ANOVA). Significant differences were set as  $p < 0.05$ . All statistical analyses were performed with the SigmaPlot 9.0, 10.0 or 13.0 software for Windows (Systat Software, Chicago, IL, USA).

## 4 RESULTS AND DISCUSSION

### 4.1 IMPACT OF DIET ON SKELETAL MUSCLE FUNCTION

In **paper I**, we investigated whether the reported claims that dietary nitrate improves athletic performance have anything to do with contractile muscle function. C57 black male mice were fed 1mM sodium nitrate in the drinking water for 7-10 days. In the first set of experiments, force measurements revealed that there is a clear increase in submaximal force in fast-twitch EDL muscle (Paper I, fig 1), but no effect on slow-twitch *soleus* muscle.

When SR  $\text{Ca}^{2+}$  release increases with increasing stimulation frequencies,  $[\text{Ca}^{2+}]_i$  will eventually reach saturation where all available cross-bridges are activated; hence the non-linear force-frequency relationship (Fig. 2). Increased submaximal force, but not maximal force, suggests that nitrate alters components affecting SR  $\text{Ca}^{2+}$  release and not total content of interacting myosin and actin in the cross-bridge cycling. Therefore, in the next set of experiments we focused on the SR  $\text{Ca}^{2+}$  release mechanism after 1 week of nitrate feeding.

Western blot analysis of EDL muscle showed that nitrate increases the SR  $\text{Ca}^{2+}$  buffering protein calsequestrin 1 (Paper I, fig 2). This indicates that there is an increased SR  $\text{Ca}^{2+}$  storage capacity in fast-twitch muscle fibers. SR  $\text{Ca}^{2+}$  release is a process of passive diffusion and hence a greater concentration gradient would lead to more  $\text{Ca}^{2+}$  released per stimulation.

To answer whether the increased SR  $\text{Ca}^{2+}$  buffering altered SR  $\text{Ca}^{2+}$  release we used real-time  $[\text{Ca}^{2+}]_i$  imaging on mechanically dissected FDB fibers. Dissected fibers have the advantage with intact tendons on each side, so the fiber can be mounted to a force transducer and injected with the fluorescent  $\text{Ca}^{2+}$  indicator indo-1. Thus  $[\text{Ca}^{2+}]_i$  and force can be measured simultaneously. With this we confirmed a shift in stimulation frequency- tetanic  $[\text{Ca}^{2+}]_i$  relationship, with greater tetanic  $[\text{Ca}^{2+}]_i$  in nitrate fed mice (Paper I, fig 3 and 4). The  $\text{Ca}^{2+}$  - force relationship follows the similar sigmoidal pattern as force-frequency relationship (Fig 3). Therefore, even small changes in tetanic  $[\text{Ca}^{2+}]_i$  will significantly affect submaximal but not maximal force. What this means is the animals will generate more submaximal force (i.e. when running) with the same mental effort as non-nitrate controls.

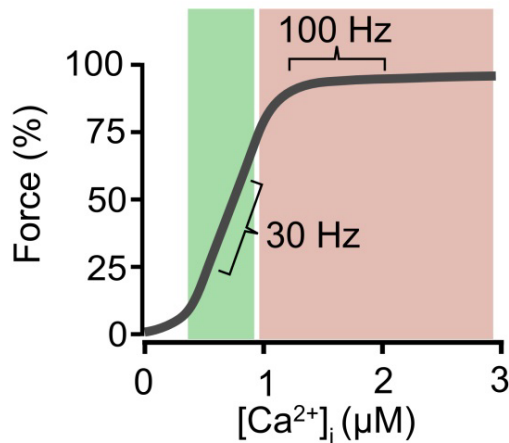


Figure 3: Diagram illustrating the force- $\text{Ca}^{2+}$  relationship. Linear part of low frequency stimulation (normal everyday activity) highlighted green. Cross-bridge saturation highlighted with red.

## 4.2 EFFECT OF SUBMAXIMAL FORCE ON VOLUNTARY ACTIVITY.

In the next study, we asked if the increased sub-maximal force would affect behavior. In theory, if the brain can lower the activity needed to generate the amount of force required for regular activity, the activity should be perceived as easier.

Here the design was similar to paper I, by which male C57 mice were given 1mM nitrate supplemented in drinking water. However, each mouse was housed individually with an in-cage running wheel (**Paper II**). The running speed and distance were recorded to measure voluntary running of each mouse. In my experience, it takes one day to a week for the mice to habituate to the wheel, however, some mice never fully use the wheel. Moreover, the surrounding condition of the room in which they are housed affect running behavior. This is important to consider in studies using voluntary running as a method for exercise or measurement of behavior. Nevertheless, the effect nitrate had on mouse voluntary running was remarkable. After 3 weeks, the nitrate fed group ran faster and further than the controls. After 6 weeks, the mice feed nitrate were covering ~60% longer distance per night than controls (Paper II, fig 2). To ensure that this was a nitrate driven effect; nitrate was replaced with regular drinking water after 6 weeks of treatment. This resulted in mice previously on nitrate consecutively decreased their running speed and distance over the following weeks. Two weeks after removal of nitrate there was no longer any statistically significant difference in running between the groups (Paper II, fig 2).

In humans, nitrate as such has previously been shown to improve mitochondrial function (Larsen *et al.*, 2011). Therefore we tested if nitrate alone, without any added exercise, affects mitochondrial function in mice. Sedentary mice fed nitrate for 3 weeks (earliest significant different in running) were tested for maximal endurance on a treadmill. The mice had to run up hill at a 25° angle, with incremental increase in speed of 2m/min every 2 minutes. This test

was set up this way based on a previous study which measured mouse oxygen consumption and carbon dioxide production in mice running at incrementally increased speed (Kemi *et al.*, 2005). They reported that the mice reach 1.0 in respiratory exchange ratio and  $\text{VO}_{2\text{max}}$  right before they were exhausted and stopped running (Kemi *et al.*, 2002).

With nitrate alone, there was no improvement in mouse endurance nor were there any improvements in mitochondrial capacity (Paper II, fig 1). There was no difference in mitochondrial respiration. Although, intriguingly, there was a decrease in mitochondrial efficiency, or amount of oxygen needed for ATP production (Paper II, fig 1). This goes in the opposite direction as that described in humans, raising a valid question as to how reliable animal models are for describing human physiology (Larsen *et al.*, 2011). However, given that mouse endurance was not affected, the reduction in mitochondrial efficiency seems to be insignificant on the grander scheme.

When nitrate was combined with 3 weeks of voluntary running, the mice treated with nitrate had better endurance on the treadmill test. Both running groups had higher activity of the mitochondrial enzyme citrate synthase (CS) than sedentary controls. Combined with nitrate, enzyme activity was even higher. However, the increased enzyme activity correlates, in a linear relationship, to the distance the mice ran. In other words, the more the mice ran; the better was the endurance (Paper II, fig 3).

If every step the mouse takes is stronger without added mental effort from the animal, each step taken would propel the mouse forward at a greater speed. Therefore running speed will increase without the mouse realizing that it runs faster. The nitrate induced enhanced submaximal force we discovered in paper I have since been shown to be true in humans as well (Haider & Folland, 2014). In terms of disease, we know that the mitochondrial myopathy model of “fast-twitch” specific *TFAM* knock-out show decreased CSQ1 and stored SR  $\text{Ca}^{2+}$ . The consequence of this is muscle weakness (Aydin *et al.*, 2009). Furthermore, PLFFD is likely caused by reduced SR  $\text{Ca}^{2+}$  release or sensitivity. Dietary nitrate might then be of interest in recommendations for people suffering from PLFFD, or the sensation of general weakness.

### **4.3 STRESS INDUCED MITOCHONDRIAL BIOGENESIS**

In **paper III** we were interested in the reports that cold exposure promoted PGC-1 $\alpha$  expression and increased mitochondrial biogenesis in skeletal muscle (Schaeffer *et al.*, 2003; Oliveira *et al.*, 2004). In cold environment, mammals shiver in order to maintain body temperature (Hemingway, 1963). Shivering causes repeated increases of  $[\text{Ca}^{2+}]_i$  which will

cause SERCA to burn more ATP in order to maintain the SR-cytosol  $\text{Ca}^{2+}$  gradient. During prolonged periods of cold exposure shivering is replaced by recruitment of uncoupling protein (UCP) dependent heat production in brown adipose tissue (Heaton *et al.*, 1978; Cannon & Nedergaard, 2004).

In initial experiments mice were acclimated in the cold ( $4^{\circ}\text{C}$ ) for 4-5 weeks. One group were UCP knockout (UCP-KO) mice, which needed to continually shiver for thermogenesis. Previously, severe RyR1 modifications and muscle weakness have been observed in *soleus* muscle from the UCP-KO cold mice (Aydin *et al.*, 2008). However, in muscles not partaking in the shivering response, e.g. FDB, there was no measurable force problem when comparing regular WT to UCP-KO (paper III, figure 1). For brevity I will therefore only focus on cold versus room temperature effects on FDB muscles. Interestingly, dissected single fibers from animals exposed to cold had higher tetanic  $[\text{Ca}^{2+}]_i$  than room temperature controls (paper III, figure 1). As described earlier, maximal tetanic  $[\text{Ca}^{2+}]_i$  has little effect on maximal force production since the cross-bridges are past  $\text{Ca}^{2+}$  saturation. However, increased tetanic  $[\text{Ca}^{2+}]_i$  brings with it interesting consequences. It would theoretically require more ATP per contraction, but it would also increase the chances of triggering the  $\text{Ca}^{2+}$  sensors (i.e. CaN, CaMKII, see Fig 4). Indeed, if we look at the baseline  $[\text{Ca}^{2+}]_i$  in between contractions we can see that it was elevated (paper III, figure 2). This increase in baseline  $[\text{Ca}^{2+}]_i$  was not due to lack of SERCA proficiency since  $[\text{Ca}^{2+}]_i$  was cleared at the same rate. Indicating a SR  $\text{Ca}^{2+}$  leak.

The following question to answer was: is the increased baseline  $[\text{Ca}^{2+}]_i$  associate with signs of increased mitochondrial biogenesis? There was a marked increase of PGC-1 $\alpha$  expression in the FDB muscles from cold exposed animals measured with Western blot. PGC-1 $\alpha$  expression was accompanied with increased *TFAM* expression and increase CS enzyme activity, indicating increased oxidative capacity (paper III, figure 3). The theoretically increased demand for ATP by SERCA was not detected by 5'-adenosinemonophosphate-activated protein kinase- $\alpha$  (AMPK) phosphorylation. This is not completely surprising since AMPK is supposedly an energy balance sensor, and according to the previous results the muscle cell has compensated for any lack of oxidative phosphorylation.

Thereafter, we tested if the increased oxidative capacity of the muscle would affect the fatigue resistance. Dissected single fibers were stimulated with 50 repeated contractions (300 ms duration) at 70Hz with 2 s intervals. Indeed, the muscle fibers from cold acclimated animals maintained  $\text{Ca}^{2+}$  release and force far better than fibers from room temperature



animals (paper III, figure 5) At the 50<sup>th</sup> contraction the cold mice still produced ~90% of initial force, whereas force in the room temperature mice were down to 40%.

It has previously been shown that cold exposure cause a minor RyR1 modification in FDB, with increased phosphorylation and decreased calstabin1 association (Aydin *et al.*, 2008). It therefore seems that the small stress from cold exposure is enough to cause a minor RyR1 dysfunction, resulting in increased baseline  $[Ca^{2+}]_i$ . This then triggers the  $Ca^{2+}$  sensors (CaN or CaMKII), which result in PGC-1 $\alpha$  expression and subsequent transcription of genes regulating mitochondrial content, volume and capacity (Fig 4). However, as seen in *soleus* muscle from the cold UCP-KO animals, too much stress is harmful. If the *soleus* muscle have to continuously shiver for thermogenesis the increased stress on RyR1 cause a severe SR  $Ca^{2+}$  leak (Aydin *et al.*, 2008). Resulting in similarities to the TFAM KO myopathy with  $Ca^{2+}$  related damage to the mitochondria, followed by reduced force (Aydin *et al.*, 2009).

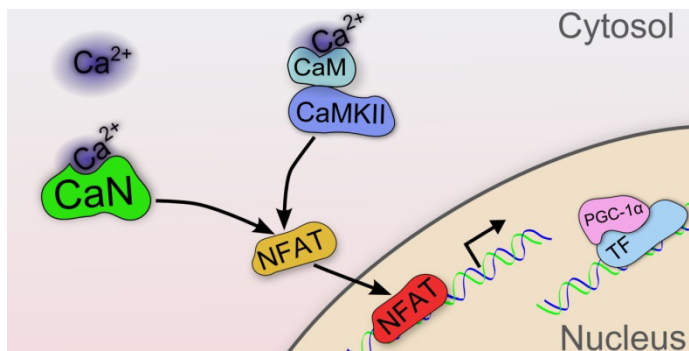


Figure 4: Illustration of proposed mechanism of how prolonged increase of baseline  $[Ca^{2+}]_i$  induces CaN/CaMKII signaling causing NFAT translocation to the nucleus resulting in PGC-1 $\alpha$  expression.

#### 4.4 THE RELATIONSHIP BETWEEN ROS, ANTIOXIDANT EFFECT AND ENDURANCE TRAINING OUTCOME.

In **paper IV** we investigated the mechanisms behind why relatively short periods of high-intensity interval training (HIIT) are more effective than regular long period low intensity exercise (Bacon *et al.*, 2013).

In the first set of experiments we studied what the effect of HIIT has on the proteins involved in excitation-contraction coupling, RyR1 in particular. A total number of 28 recreationally active human subjects were recruited to perform one session of HIIT, in the form of repeated Wingate tests. The exercise consisted of 30 s all-out cycling bouts at 0.7 Nm/kg body weight on a cycle ergometer, with 4 min rest between tests. One group repeated the set three times, another group six times. Muscle biopsies were taken from *vastus lateralis* from the subjects which performed six repeated Wingate tests before, after and 24 hours after the test. Force

production and EMG signals were also measured before and up to 24 hours after exercise (Paper IV, SI appendix fig S1).

Voluntary maximal contraction (MVC) and femoral nerve stimulated contraction were decreased for hours following the HIIT exercise. Even 24 hours after the exercise there was a small component of PLFFD. The most remarkable finding was the fragmentation of RyR1 observed 24 hours after HIIT exercise. RyR1 is a homeotetramere and each subunit is ~565kDa large each (Zalk *et al.*, 2015). After HIIT we observed a decrease in the full length RyR1, and instead emergence of 375, 80 and 60 kDa size peptides from the c-terminal end (Paper IV, fig 1). Noteworthy, the pore-forming domain of RyR1 is predicted to be at the ~15kDa c-terminal end (fig 5). Since the subjects were showing no major force problems 24 hours after the exercise, it is likely that this fragmented state of RyR1 can still function as a  $\text{Ca}^{2+}$  channel.



*Figure 5: Illustration of how the size predicted RyR1 fragments line up with the RyR1 - peptide sequence (uniprot.org/uniprot/P21817).*

If we compare HIIT to a prolonged relatively low intensity, but still greatly energy consuming form of exercise, namely marathon running, we did not observe any increase in RyR1 fragmentation. What we discovered instead was a dissociation of the stabilizing molecule calstabin1 from RyR1 (Paper IV, SI appendix fig S2). Others have shown that when calstabin1, or FKBP12 as it also is referred to, is dissociated from RyR1, the open probability of RyR1 increases, which means it becomes 'leaky' (Ahern *et al.*, 1997; Bellinger *et al.*, 2008). Our current presumption is that this dissociation also occurs with fragmented RyR1 from the HIIT subjects. Unfortunately, the method of immunoprecipitation necessary to

measure level of RyR1-calstabin1 association requires an equal antibody to epitope affinity between samples. The end result of my attempt at RyR1 immunoprecipitation on HIIT was a decrease in both calstabin1 and RyR1 signal (data not shown). Regardless, there are other methods for determining HIIT effect on  $\text{Ca}^{2+}$  release and  $[\text{Ca}^{2+}]_i$ , which I will come back to later.

We also focused on the discrepancies between the relatively great benefit of HIIT for non-athletes (Bacon *et al.*, 2013), but rather modest to no difference for elite athletes (Guellich *et al.*, 2009; Laursen, 2010). Therefore, we replicated the same Wingate HIIT exercise, but with elite endurance athletes (average  $\text{VO}_{2\text{max}}$  of 67 ml/min/kg). The elite athletes fatigued themselves to similar force depression as the recreationally active participants (Paper IV, SI appendix fig S10). However, no RyR1 fragmentation was observed after HIIT in elite athletes (Paper IV, figure 2). PGC-1 $\alpha$  mRNA expression after HIIT was immediately increased for both the recreationally active and elite athletes, however, interestingly, for the recreationally active PGC-1 $\alpha$  falls below pre condition after 24 h (Paper IV, figure 2). This suggests an overcompensating feedback loop. Calcineurin for instance, is known to have one such self-transcribed inhibitor, calcipresin, which prevents calcineurin from dephosphorylating NFAT (Rothermel *et al.*, 2003).

The next question we addressed was what causes the RyR1 fragmentation. Furthermore, why did this not occur in the athletes? Since RyR1 is known to be susceptible to various ROS/RNS modifications, we wondered if the athletes simply were better protected against ROS/RNS. Indeed, the antioxidant enzymes of SOD2 and catalase were higher in elite athletes than in recreationally active individuals, presumably due to their greater oxidative capacity, which includes greater endogenous protection against ROS/RNS (Paper IV, figure 2). This is interesting since it has been shown that supplementation with strong antioxidants can hamper some of the positive exercise response (Ristow *et al.*, 2009; Paulsen *et al.*, 2014).

Which begs the question, is ROS/RNS production during HIIT an important step for the RyR1 fragmentation and the final cellular response? For the next set of experiments we therefore replicate HIIT exercise in isolated mouse muscles. The problem with isolated muscles is that there is no normal blood flow through capillaries, which creates an oxygen diffusion problem to the core of the muscle (Zhang *et al.*, 2006b). To avoid this problem, we divided up FDB muscles into individual digits. These muscles are relatively flat, so most of the muscle fibers would be directly exposed to the oxygenated Tyrode, reducing the risk of hypoxia.

The ‘simulated’ Wingate protocol increased MDA modifications on RyR1 5 minutes after the last stimulation, in mouse FDB single digits (Paper IV, fig 3). Three hours later, RyR1 was fragmented, similar to what was seen with HIIT in the human subjects. Intriguingly, presence of the ROS scavenger *N*-acetylcysteine (NAC) during stimulation and recovery prevented the RyR1 fragmentation (Paper IV, fig 3). Clearly, ROS/RNS modification of RyR1 seems necessary for RyR1 fragmentation. However, how does this post-translational modification cause proteolysis? Normal protein degradation is either handled indiscriminately by the lysosome, or targeted by ubiquitination for proteasome proteolysis, or targeted by specialized enzymes with unique interaction. We could not detect any increased levels of ubiquitinated proteins in the human HIIT subjects (Paper IV, Si appendix fig S3). Furthermore, since only RyR1 seemed to be affected, neither lysosome nor proteasome degradation seems to fit the rather specific cleavage of RyR1. However, it previously been shown that RyR1 can be a substrate for calpain-3, a muscle-specific member of the calpain family of non-lysosomal  $\text{Ca}^{2+}$ -dependent proteases (Sorimachi *et al.*, 1989; Shoshan-Barmatz *et al.*, 1994; Shevchenko *et al.*, 1998). We measured total calpain activity of mouse muscles stimulated with HIIT and observed an increase in activity already 30 minutes after last contraction. The calpain-3 therefore seems to be part of the mechanism for HIIT induced RyR1 fragmentation. Another interesting aspect of calpain-3 is that mutations resulting in aberrant enzyme function are linked to limb girdle muscular dystrophies (Vainzof *et al.*, 2003). Hence, suggesting the mechanism of  $\text{Ca}^{2+}$  induced calpain is important for healthy muscles.

Furthermore, after calpain-3 has cleaved RyR1, the channel becomes stabilized in an open sub conducting state (Shevchenko *et al.*, 1998), i.e. the remaining pore forming domain is ‘leaky’. The remaining question was then whether the HIIT induced modifications of RyR1 causes changes in SR  $\text{Ca}^{2+}$  handling. We therefore decided to scale the ‘simulated’ Wingate down to the single fiber level. Single fibers were mechanically dissected from mouse FDB muscles in the same manner as described for Paper I and mounted to a force transducer and injected with the fluorescent  $\text{Ca}^{2+}$  indicator indo-1. The fibers were then stimulated with the same ‘simulated’ Wingate protocol as the mouse toes’

There was a marked reduction in tetanic  $[\text{Ca}^{2+}]_i$  between the first contraction of the first set and the first contraction of the last set. The fibers presented marked force decrease, both at high and low stimulation frequencies for up to 2 hours following stimulation. This force depression was due to a reduced amount of tetanic  $[\text{Ca}^{2+}]_i$ . Intriguing, baseline  $[\text{Ca}^{2+}]_i$  was increased throughout the recovery period. Measurements using caffeine (an RyR1 agonist), which forces the RyR1 channel to fully open, indicated that there was less stored  $\text{Ca}^{2+}$  in the

SR (Paper IV, fig 4). Presumably both due to that a large proportion of the cells  $\text{Ca}^{2+}$  is located in the cytosol and the formation of  $\text{CaP}_i$  precipitates.

It would thus appear that HIIT causes a very severe modification of RyR1, which presumably takes a long time to repair. The prolonged increase in baseline  $[\text{Ca}^{2+}]_i$  would put the muscle cell under increased metabolic stress because of the increased ATP consumption by SERCA trying to maintain the SR-cytosol  $\text{Ca}^{2+}$  gradient. Intriguingly, 18-22h after a single bout of high-intensity training, rest lipid oxidation is increased by ~60% in humans (Whyte *et al.*, 2013). Therefore we performed a small related experiment that was not originally part of paper IV. Mouse *soleus* muscles were repeatedly stimulated to a similar state of force depression as the HIIT stimulated FDB muscles (two per second, 50Hz, 100ms long contractions for 800 contractions). The *soleus* muscles were then incubated in oxygenated Tyrode for 20 hours. Thereafter basal glucose uptake was measured, which showed an increased metabolic demand (fig 6). This data indicates that HIIT causes a prolonged SR  $\text{Ca}^{2+}$  stress over at least 24h, which is plenty of time for the  $\text{Ca}^{2+}$  sensors (CaN and CaMKII) to react and signal subsequent transcription of factors necessary for mitochondrial biogenesis.

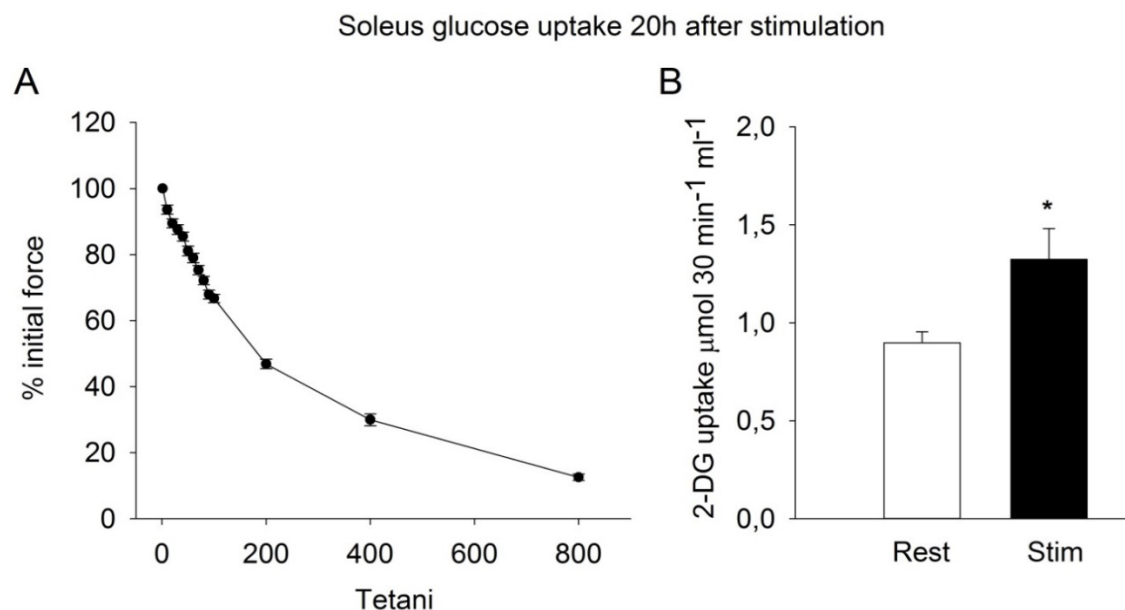


Figure 6: Soleus muscle stimulated to ~20% fatigue (A) incubated overnight in oxygenated Tyrode solution followed by glucose uptake measurements (B), \* $p < 0.05$  with *t*-test,  $n = 8-10$ .

#### 4.5 THE LINK BETWEEN EXERCISE, BASELINE $[Ca^{2+}]_i$ AND MITOCHONDRIAL BIOGENESIS.

In **paper V** we wanted to closer examine the role of SR  $Ca^{2+}$  leak and increased muscle endurance. As observed in paper III, prolonged periods of mild stress by cold-acclimation induced a small SR  $Ca^{2+}$  leak which remarkably causes increased fatigue resistance in mouse FDB muscles. In line with this, paper IV showed that both a single session of either prolonged low intensity (marathon running) or short high intensity (HIIT) exercise produce some level of RyR1 modification resulting in a prolonged SR  $Ca^{2+}$  leak similar to that of the cold acclimated mice.

For the first set of experiments we assessed what effect voluntary running has on RyR1 and baseline  $[Ca^{2+}]_i$ . Similar as described for paper II, mice were housed individually with an in-cage with a running wheel. Sedentary controls were housed with a locked wheel. Running distance incrementally increased for the first three week, after which the mice entered a form of steady state running distance of ~12 km per night (Paper V, figure 1). There was no difference in body weight between runners and sedentary controls, although, there was a visible difference in fat pad size between the two. Since the mice reached this “steady state” running distance at three weeks, we chose this time point for the following experiments.

There was a slight tendency for increased force per cross sectional area in single muscle fibers from the runners, but the data was not statistically significant. In addition, no difference in tetanic  $[Ca^{2+}]_i$  was observed over the range of stimulation frequencies (15-150 Hz). However, similar to that observed in the cold acclimated animals, baseline  $[Ca^{2+}]_i$  was increased by ~30%. When the fibers were subjected to a fatiguing protocol of repeated stimulations, the fibers from the runners were strikingly more fatigue resistant than the sedentary control (Paper V, fig 2).

We then analyzed *tibialis anterior* (TA) muscles from these mice and observed a decreased calstabin1 association with RyR1, indicating leaky channel. Moreover, there was an increased PGC-1 $\alpha$  protein expression, which suggest an ongoing signaling for mitochondrial biogenesis. The muscles also expressed more of mitochondrial electron transport chain protein cytochrome c oxidase, subunit 1 (COX1), and increased enzyme capacity of both citric acid cycle enzyme CS and  $\beta$ -oxidation protein HAD (Paper V, fig 3). To sum up, voluntary running causes a RyR1 leak, which raises the baseline  $[Ca^{2+}]_i$ , which causes increased mitochondrial biogenesis and improved mouse exercise endurance.

After 6 weeks of running, when there had not been any increase in running distance for three weeks, there was no indication of destabilized RyR1, presumably because the mice were no longer increasing their running intensity. Consequently, PGC-1 $\alpha$  protein had stabilized to normal levels, suggesting no further signaling for more mitochondria. However, since the mice were still running, they maintained their elevated content of mitochondrial protein COX1 (Paper V, fig 4). This suggests the muscle fibers adapted to the first three weeks of SR Ca<sup>2+</sup> stress, because of the increased mitochondrial capacity they were no longer affected by the metabolic stress of running ~12km per night.

Based on our findings in Paper III, IV and V, RyR1 leak and increased baseline [Ca<sup>2+</sup>]<sub>i</sub> seems to be a common feature in regards to endurance exercise. Question remains if it is just a side effect or a relevant step in signaling for improved endurance.

In the next set of experiment, we tested if pharmacologically induced RyR1 leak could mimic exercise. It has previously been shown that rapamycin can dissociate calstabin1 from RyR1 in a dose dependent manner (Ahern *et al.*, 1997). It has also been reported that injecting low doses of rapamycin, far below normal therapeutic dose, increases protein synthesis (Lee *et al.*, 2014a). Therefore, we injected mice with a concentration of 1ng rapamycin per g bodyweight every 48h hoping to mimic a “regular human exercise pattern” (i.e. going to the gym to run on a treadmill every other day after work). The hypothesis is that rapamycin would destabilize RyR1 and cause a prolonged increase in baseline [Ca<sup>2+</sup>]<sub>i</sub> over the following hours, similar to that seen with running exercise (for both in human and mouse).

After three weeks of treatment, we tested the animals’ endurance using the same treadmill endurance test as was used in paper II. Sedentary mice injected with rapamycin had a small but significant increase in treadmill endurance compared to sedentary saline controls. Rapamycin had the desired effect of creating a small and dissociation of calstabin1 from RyR1 (Paper V, Fig 5), which indicates that a small leak has been introduced. This leak, similar to that of the regular runners, could be seen as an increase in baseline [Ca<sup>2+</sup>]<sub>i</sub> (Paper V, Fig 5). We could also detect a very small ~10% increase in PGC-1 $\alpha$  protein, together with improvements in CS and HAD enzyme activity (Paper V, Fig 5). Thus pharmacologically inducing a leaky RyR1 can mimic events induced by voluntary running.

The presented results fit with previous studies done in L6 myotubes, where inducing calmodulin kinase activity or incubating with caffeine, which also increases baseline [Ca<sup>2+</sup>]<sub>i</sub>, induced mitochondrial biogenesis (Wu *et al.*, 2002; Ojuka *et al.*, 2003). There is also the interesting peculiarity of *ATCN3* (R577X) mutation, which leads to  $\alpha$ -actinin-3 deficiency.

The frequency of the mutation correlates with greater latitude and lower temperatures (Friedlander *et al.*, 2013). It is also surprisingly prevalent in endurance athletes, but underrepresented in elite sprinters (Vincent *et al.*, 2007). What is the truly interesting part about  $\alpha$ -actinin-3 deficiency is that ATCN3 KO mice have increased baseline  $[Ca^{2+}]_i$  (Head *et al.*, 2015). What this shows is that increased skeletal muscle baseline  $[Ca^{2+}]_i$  is not only a common phenomenon in exercise or other form of stress, it is also part of the signaling towards mitochondrial biogenesis.

Nonetheless, some question still remains, e.g. what are  $Ca^{2+}$  interacting with in order to improve mitochondrial biogenesis, calcineurin or CaMKII being the two likely candidates (Wu *et al.*, 2002; Tavi & Westerblad, 2011). Noteworthy, “exercise in a pill” or pharmacological exercise have been performed before, by the use of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) to stimulate AMPK, resulting in minor increases of mitochondrial proteins and the endurance of sedentary animals (Narkar *et al.*, 2008). AMPK however, can be activated by  $[Ca^{2+}]_i$ , so perhaps  $Ca^{2+}$  induced AMPK activation also is a relevant pathway (Witczak *et al.*, 2008). Although, in paper III we did not see any indication of increased AMPK phosphorylation (paper III, fig 4).

What is even more interesting is when we combined rapamycin injection with voluntary running. The mice did not differ in running distance whether they were injected with rapamycin or regular saline. However, the rapamycin injected runners performed better on the treadmill endurance test than the regular runners, and had a higher CS capacity. Thus, rapamycin introduced an additive effect of exercise. Lee *et al.* showed a similar effect: when mice were trained on a treadmill every day, the rapamycin injected mice improved their endurance capacity faster (Lee *et al.*, 2014a).

As we observed with the difference between HIIT and marathon, the exercise which is most effective at promoting mitochondrial biogenesis, also happens to cause the greatest RyR1 modification. Presumably, contrary to HIIT, mice voluntary running does not occur at or beyond the mouse maximal aerobic capacity. I then propose that there is extra room in terms of further increased SR  $Ca^{2+}$  leak which will still be stressful enough to add to the mitochondrial biogenesis signaling, but still not stressful enough to cause the  $Ca^{2+}$  linked damage seen in for instance overtraining (Bellinger *et al.*, 2008).

On an unrelated peculiarity, western blot of the mice which have been running for six weeks showed an increase in RyR1 total protein, but not in CSQ1 or DHPR (Figure 7). Mice fed dietary nitrate had increased CSQ1 and DHPR but not RyR1 (Paper I, figure 2). This



indicates that, despite these protein are all part of the same cellular mechanism, signaling for their transcription are differ.

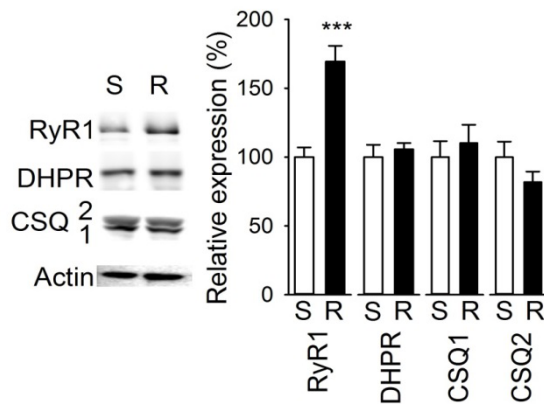


Figure 7: Western blot for RyR1, DHPR, CSQ1 and 2 of sedentary mice (S) or mice given access to an in-cage running wheel (R) for six weeks. \*\*\*  $p < 0.001$  with  $t$ -test,  $n=6$ .

#### 4.6 MITOCHONDRIAL DYSFUNCTION AND SR $\text{Ca}^{2+}$ RELEASE RELATED MYOPATHY.

For the next study we were interested in the mechanisms behind mitochondrial myopathy, particularly myopathy with ageing.

RyR1-mediated SR  $\text{Ca}^{2+}$  leak has been shown to be a feature in ageing (Andersson *et al.*, 2011). We also know that the mitochondria can take up  $[\text{Ca}^{2+}]_i$ , and excessive uptake can be harmful (Aydin *et al.*, 2009; Gineste *et al.*, 2015). Can the mitochondria sense a  $\text{Ca}^{2+}$  overload and initiate signaling to reduce SR  $\text{Ca}^{2+}$  release, resulting in muscle weakness? Findings from TFAM KO mice, suggests this. The TFAM KO is a mouse model where the muscle cannot form new mitochondria once it reaches adult age. The ageing mitochondria left are forced to struggle with in increased stress. Intriguingly, if the mitochondria are prevented from absorbing  $\text{Ca}^{2+}$ , the muscle weakness can be prevented (Gineste *et al.*, 2015).

In the last study (**Paper VI**) we examined the relationship between, ageing, degenerating mitochondria, and SR  $\text{Ca}^{2+}$  release mechanism. For this we used a mouse model for premature ageing, namely the PolgA mtDNA mutator mouse model. These mice have been engineered to lack the proof reading mechanism for mtDNA replication. Unlike the TFAM KO model, which cannot form new mitochondria at an adult age, mtDNA mutators will regenerate progressively worse mitochondria resulting in rapid ageing, where 11 month old mice have the comparable phenotype of a 30 month old wild-type (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005).

In the first set of experiments we wanted to establish when age related problems started to occur in the mtDNA mutator mice. Around 3-5 month of age we could not detect any problems with force production or SR  $\text{Ca}^{2+}$  release in either EDL muscles or single FDB fiber (Paper VI, figure 1). However, there were already an energy supply problem; decreased mitochondrial enzyme activity of both CS and HAD as well as reduced COX1 protein (Paper VI, figure 3). This was accompanied by decreased fatigue-resistance in single fibers from mtDNA mutator mice as compared to healthy wild-type controls (Paper VI, figure 2). This demonstrates that the progressive mitochondrial problem starts to occur before the onset of myopathy. There also seemed to be a drive for reduced mitochondrial biogenesis as PGC-1 $\alpha$ , AMPK and PPAR $\alpha$  were reduced in mtDNA mutator mice already at a young age (Paper VI, figure 5).

In the second set of experiments we measured the outcome of this energy supply problem. At 11 month of age the mtDNA mutator mice displayed force problems, which was the result of reduced tetanic  $[\text{Ca}^{2+}]_i$  (Paper VI, figure 6). Western blot data indicated that the reduced tetanic  $[\text{Ca}^{2+}]_i$  was not due to the  $\text{Ca}^{2+}$  release mechanism, but rather the SR  $\text{Ca}^{2+}$  pump, SERCA1 (Paper VI, figure 7). Thus, the muscle solves the mitochondrial energy production problem by signaling for a reduction in one of the greatest ATP consumers in the skeletal muscle.

A second interesting factor is that in the TFAM KO mice, nuclear produced parts of the oxidative phosphorylation machinery such as CS are increased, presumably to compensate for the degrading mitochondria (Wredenberg *et al.*, 2002). In the mtDNA mutator the opposite occurred. What remains unknown is why a mitochondrial energy problem result in a reduced mitochondrial biogenesis? Can the skeletal muscle sense that it is generating bad mitochondria? To further add to the mystery of mtDNA mutators, endurance exercise still improves mitochondrial biogenesis and mitigates the phenotype of advanced ageing (Safdar *et al.*, 2011). While the health of mitochondria seems to have a possibility to determine the skeletal muscle function, the nucleus seems able to dictate the general health of mitochondria. We just have to activate that signaling cascade. If the mDNA model for ageing can be translated to human physiology, this is a good sign for the ageing population. If activating once muscle can prevent, or at least alleviate some, age related fatigue, other age related diseases might be mitigated as well.

## 4.7 CONCLUDING REMARKS

It is no secret that oxidative capacity and endurance exercise are important for good general health. As mentioned in the introduction, a sedentary lifestyle is associated with ‘western world’ diseases, while an active lifestyle reduces the risk of mortality and generally improves quality of life. If the mtDNA mutator model in study VI is to be believed, there is a bleak outlook for us sedentary people if we do not exercise and stimulate our mitochondria.

However, the major hurdle, as the author himself can attest to, is to stay activate; what is loosely referred to as ‘fatigue’ either disincentives, or prevent physical activity. As we could see in study I and II, dietary nitrate can alleviate the sensation of ‘fatigue’, and of personal observation it does make mundane moderate activity such as walking up stairs easier. What we can see from study III and V is that for us lazy people, there is hope. While exercise, especially the really intense form seen in study IV still are the most effective methods to improve oxidative capacity. Perhaps in the future, using mechanisms such as controlled SR  $\text{Ca}^{2+}$  leak could be a helpful tool, at least in pathological conditions where normal movements are hindered.

## 5 ACKNOWLEDGEMENTS

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